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(54) Title: METHODS FOR SCREENING COMPOUNDS USING ENCAPSULATED CELLS

(57) Abstract

The present invention relates to an integrated approach to drug screening that is designed to couple a screening assay both temporally and spatially to natural product synthesis in a microorganism. The present invention provides a screening unit which is a gel droplet comprising a producing species that produce natural products for the drug screen, and an assay system that detects or measures a desired biological activity. A producing species is coencapsulated with an assay system in a screening unit when the producing species is at a phase in its life cycle that is optimal for producing natural products, such as secondary metabolites. The producing species is spatially positioned relative to the assay system in the same unit such that compounds produced by the producing species can come into contact with the assay system. If a compound possesses the desired activity, the assay system will generate a signal that enables the identification and/or isolation of the screening unit. The present invention also provides methods for forming a screening unit, and methods for using a screening unit in drug screening. The methods are useful for screening naturally occurring organisms as well as genetically engineered cells, and solid carriers containing chemicals.

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METHODS FOR SCREENING COMPOUNDS USING ENCAPSULATED CELLS

1. FIELD OF THE INVENTION

The present invention relates to an integrated approach to drug screening. More particularly, the invention provides methods for culturing and encapsulating microorganisms such that the production of secondary metabolites by the microorganisms are temporally and spatially coupled to a screening assay. The methods of the invention enable the optimal production of compounds of potential interest and the rapid determination of desired biological activities within the same environment. The methods are useful for screening naturally occurring organisms as well as genetically engineered cells. The encapsulated cells also have utility in commercial production of the compounds.

2. BACKGROUND OF THE INVENTION

2.1. DRUG DISCOVERY

The basic challenges in drug discovery are to identify a lead compound with the desirable activity, and to optimize the lead compound to meet the criteria required to proceed with further drug development.

Traditionally, the source of potential drug candidates are collections of natural products. Identification of lead compounds has been achieved by random screening of such collections which encompass as broad a range of structural types as possible. Random screening of natural products from sources such as terrestrial bacteria, fungi, invertebrates and plants has resulted in the discovery of many important drugs (Franco et al. 1991, Critical Rev Biotechnol 11:193-276; Goodfellow et al. 1989, in "Microbial Products: New Approaches", Cambridge University Press, pp. 343-383; Berdy 1974, Adv Appl Microbiol 18:309-406; Suffness et al. 1988, in Biomedical Importance of Marine Organisms, D.G. Fautin, California Academy of Sciences, pages 151-157). More than 10,000 of these natural products are biologically active

and at least 100 of these are currently in use as antibiotics, agrochemicals and anti-cancer agents.

The recent development of synthetic combinatorial chemical libraries and libraries of genetically engineered microorganisms will increase the number and variety of compounds available for screening.

One common approach to drug discovery involves testing whole cells or organisms that are representative of the causative agent of the disease in bioassays in which potential drug candidates are assayed for therapeutic activity. Another approach utilizes large numbers of individual biochemical assays in which the activity of a drug target, such as an enzyme, a receptor or a transcription factor, is determined in the presence of the potential drug.

The success of this approach of drug discovery depends on assay throughput, number and diversity of compounds, number of screens running in parallel, and the ability to discriminate meaningful activity from false positives and background noise. Typically, pharmaceutical companies screen compound collections containing hundreds of thousands of natural compounds. However, the ratio of novel to previously-discovered compounds has diminished with time. Partly, this is due to the difficulties of consistently and adequately finding, reproducing and supplying novel natural product samples.

With the advent of genomics and the understanding of the molecular basis of diseases, a large number of novel drug targets, and mechanism-based screens will emerge and be tested against the natural and synthetic libraries of compounds. There is a probability that sample diversity as well as the screening process will become a rate limiting step in a drug discovery program. Thus, there is also a demand for screening processes that are efficient and amenable to automation so as to reduce what was once a slow process to one that is quick and systematic.

Immobilization of cells is a relatively modern tool in biotechnology for investigation into microbial processs.

This technique has been used in the production of several antibiotics, such as penicillins (1984, Deo et al., Biotechnol Bioeng, 26:285-295; 1990, Flanagan et al., Biotechnol Bioeng, 36:608-616), bacitracin (1980, Morikawa et al., Biotechnol Bioeng, 22:1015-1023), erythromycin by *Streptomyces erythreus* (1993, Bandyopadhyay et al., Biotechnol Letters, 15:1003-1006), and oxytetracycline by *Streptomyces rimosus* (1994, Farid et al., 3:301-309). The technique has also been explored for use in biotransformation, transplantation, clinical microbiology, toxicology, food chemistry and the environmental sciences. See for example, O'Reilly et al. (1995, Enzyme Microb. Technol. 17:636-646) U.S. Patent Nos. 4,399,219; 4,401,755; 4,634,968; 4,916,060; 4,959,301; 5,055,390; 5,225,332; and 5,578,314.

2.2. SECONDARY METABOLITES AS SOURCES OF DRUGS

Microorganisms, fungi, invertebrates and plants have historically been used as sources of natural products for drug screening. For example, fungi are an extremely diverse group of heterotrophic microorganisms that are used for the production of foods, enzymes, and pharmaceuticals. Filamentous fungi grow as branched filaments and produce a vast array of secondary metabolites.

Secondary metabolites are natural products produced by microorganisms which are not essential for vegetative growth of the organism in nutrient-rich culture. These compounds are often medicinally active, and usually possess unusual chemical structures and linkages such as β-lactam rings, cyclic peptides comprising normal and modified amino acids, unsaturated bonds of polyenes and polyacetylenes, and macrolide rings (1992, Demain, in Biotechnology of Filamentous Fungi, ed. Finkelstein and Ball, Chapter 5, pages 89-112, Butterworth-Heineman). Secondary metabolites generally have survival functions in nature and are produced as a mixture of members of a chemical family because of the

low specificity of some enzymes involved in the secondary metabolism.

Traditionally, the strategies used for screening microorganisms for drug leads have include primarily, random mutation and selection. Recombinant DNA technology has recently been applied to identify genes involved in the biosynthetic pathways that produce secondary metabolites and specialty chemicals. These pathways require multiple enzymes encoded by genes that are clustered in the genome, for example, genes encoding pathways of bacterial polyketide syntheses (PKSs) (Malpartida et al. 1984, Nature 309:462; Donadio et al. 1991, Science 252:675-679). PKSs catalyze multiple steps of the biosynthesis of polyketides, an important class of therapeutic compounds, and control the structural diversity of the polyketides produced. For example, a host-vector system in *Streptomyces* has been developed that allows directed mutation and expression of cloned PKS genes (McDaniel et al. 1993, Science 262:1546-1550; Kao et al. 1994, Science 265:509-512). This specific host-vector system has been used to develop more efficient ways of producing polyketides, and to rationally develop novel polyketides (Khosla et al., WO 95/08548).

3. SUMMARY OF THE INVENTION

The present invention relates to an integrated approach to drug screening. The invention provides methods for culturing and encapsulating microorganisms that enable the optimal production of natural products of potential interest and the rapid determination of desired biological activities.

The invention is based, in part, on the recognition that secondary metabolites production can be suppressed by high specific growth rates of the producing cultures. The time of appearance of the enzymes and the formation of individual biosynthetic pathways for natural products, such as secondary metabolites, depend on many factors, such as induction, nutrient repression, synthetase decay and end-product

regulation. The screening methods of the invention are designed to couple the screening assay both temporally and spatially to natural product synthesis in a microorganism.

In one embodiment, the present invention provides a screening unit for use in a drug screening assay, which is a gel droplet comprising a producing species that produce compounds of interest, and an assay system that detects or measures a desired biological activity. The producing species in a screening unit comprising an assay system is spatially positioned relative to the assay system in the same unit such that compounds produced by the producing species can come into contact with the assay system. If a compound possesses the desired activity, the assay system will generate a signal that enables the identification and/or isolation of the screening unit.

The present invention also provides methods for forming a screening unit, and methods for using a screening unit. In various embodiment of the invention, a producing species is incorporated into a screening unit when the producing species is at a phase in its life cycle that is optimal for producing secondary metabolites. The methods can be used with naturally occurring organisms as well as genetically engineered cells. In another embodiment of the invention, where a producing species is a solid carrier containing one or more compounds, the producing species is incorporated into a screening unit and placed under the appropriate conditions that trigger release and presentation of the compounds to the assay system in the screening unit.

The assay system of the invention comprises reporter molecules, reporter regimens, or live indicator cells or organisms which may optionally comprise a target molecule of the desired compound and/or a reporter molecule.

In specific embodiments, the method of the invention comprises the following steps in the order stated: culturing a producing species for an interval under culture conditions such that production of secondary metabolites begins; encapsulating the microorganism together with an assay system

in a gel matrix to form a screening unit; culturing the unit for an interval sufficient for the secondary metabolites produced by the producing species to contact the assay system; and detecting a signal generated by the assay system.

In another specific embodiment, the method of the invention comprises the following steps in the order stated: encapsulating a producing species in a gel matrix to form a gel droplet; culturing the gel droplet for an interval under culture conditions such that production of secondary metabolites begins; forming around the gel droplet (an outer layer of) a second gel matrix comprising an assay system to form a screening unit; culturing the screening unit for an interval sufficient for secondary metabolites produced by the producing species to interact with the assay system; and detecting a signal generated by the assay system.

The encapsulated cells also have utility in commercial production of the compounds, and in conventional screening assays.

4. DESCRIPTION OF THE FIGURES

Figure 1A-1B. Coencapsulation of producing organism and target organism. Figure 1A: Schematic diagram showing single layer coencapsulation of producing species and assay system or indicator. Figure 1B: Schematic diagram showing double layer encapsulation where the producing species is in the inner region and the assay system or indicator organism is in the outer region.

Figure 2. Antimicrobial activity assay. Double layer encapsulation of *Streptomyces parvulus* (left) with *Enterococcus faecalis* indicator cells in the outer layer; and *Streptomyces lividans* (right) in the inner layer with *Enterococcus faecalis* indicator in the outer layer. Only S. *parvulus* showed antimicrobial activity.

Figure 3A-B. Induction of a dinD promoter activity and β-galactosidase gene expression in *E. coli* coencapsulated with *Streptomyces*. Fig. 3A: No induction of β-galactosidase

gene expression in *E. coli* cells. Figure 3B: Blue colonies indicate induction of β-galactosidase expression in *E. coli* cells due to the presence of a DNA-damaging agent produced by the coencapsulated *Streptomyces*.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an approach to drug discovery that integrates the production of potential drug candidates with a high throughput screening assay. More particularly, the invention provides methods for culturing and encapsulating cells (such as bacteria, yeast, fungi, plant, and mammalian cells) and methods for encapsulating chemical compounds on solid carriers such that the production or presentation of compounds of potential interest are temporally and spatially coupled to a screening assay. The methods of the invention allow production of natural products by microorganisms or genetically engineered cells that are good or promising sources of drugs under optimal conditions, while the compounds produced are tested simultaneously for a desired activity by the coupled assay system. The present invention facilitates the very efficient screening of a large number of compounds using live cells.

The invention is based, in part, on the recognition that secondary metabolite synthesis is generally suppressed by high specific growth rates of the producing cultures. Expression of the genes controlling secondary metabolite synthesis does not usually occur at high growth rates due to repression of the syntheses during growth. The time of appearance of the enzymes and the formation of individual biosynthetic pathways for secondary metabolites depend on many factors, such as induction, nutrient repression, synthetase decay and end-product regulation. In batch cultures containing the appropriate or nutritionally rich media, high levels of secondary metabolites are produced usually only after most of the cell growth has been completed. The screening methods of the invention are

designed to couple the presence of the screening assay both to the time when secondary metabolite synthesis begins in the microorganism.

In one embodiment, the present invention provides a discrete unit of screening, which is a gel droplet comprising at least one producing species and at least one assay system. The producing species in a screening unit is spatially positioned relative to an assay system in the same unit such that compounds produced or presented by the producing species can come into contact with the assay system.

As used herein, a producing species is any cell that is capable of producing a natural product that can be a potential drug candidate. A solid carrier of chemical compounds that presents the compounds to an assay system can also be a producing species. Descriptions of producing species are provided in detail in Section 5.2.

The term "compound" refers to a compound or natural product, including secondary metabolite, produced by a producing species which is to be tested by assay systems of the invention for a particular desired activity. The compounds of the invention encompass numerous classes of chemical molecules, though typically they are organic molecules, and preferentially of low molecular weight. Such compounds comprise functional chemical groups necessary for the desired activity, such as but not limited to cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more functional groups, including but not limited to alkyl, carbonyl, amine, hydroxyl or carboxyl groups. The compounds of the invention are not limited to natural products, such as secondary metabolites, but also encompasses enzymes and structural components of an organism. A compound may serve as an agonist or an antagonist to a class of receptor or a particular receptor. A compound of interest may have one or more potential therapeutic properties, including but not limited to antibiotic, antiviral, antitumor, pharmacological, or immunomodulating properties, or other commercially-

valuable uses, e.g., agrichemicals, pesticides for crop protection, pigments, or industrial enzymes.

An assay system refers to any means that facilitates detection or measurement of a desired activity, such as but not limited to bactericidal activity, bacteriostatic activity, receptor binding, growth inhibition, etc. Detailed descriptions of assay systems are provided hereinbelow in Section 5.3.

Due to the permeable nature of the gel matrix, compounds produced by the producing species in one gel region can diffuse throughout the screening unit. If a compound possesses the desired activity, the assay system will generate a signal that enables the identification and/or isolation of the screening unit.

In other embodiments of the invention, methods for forming a screening unit and methods for using a screening unit are also provided.

It is known that immobilization conditions can affect the production levels as well as the kind of secondary metabolites produced by immobilized microorganisms (Roisin et al., Ann NY Acad Sci 782:61-69). Therefore, the inventors set out to identify factors that are important in building a drug screening process which is based on cell encapsulation. In accordance with the invention, the following factors should be taken into account in the design of such a drug screening process: (a) the physical size and form of the producing species; (b) the environmental and nutrient requirements of the producing species for production and secretion of secondary metabolites; (c) the biochemical or biological nature of the assay; and (d) the operating parameters of the assay. For example, the growth potential and accompanying morphological changes of the producing species or indicator cells (as an assay system or a component thereof) can significantly affect the integrity of the gel droplet.

In particular, in the life cycle of a microorganism, there is a growth phase and a production phase. The two

phases are distinct in microbial cultures growing in complex media capable of supporting rapid growth. The two phases may overlap in culture media that support slow growth. To maximize the efficiency of the drug screening process, the time of secondary metabolite production and the time of coencapsulation with an assay system should be synchronized such that the assay system is in place just when secondary metabolites are produced. Thus, depending on the biology of the producing species, and the type of activity sought after in the screen, the methods for forming and using the screening unit of the present invention may vary accordingly.

In one embodiment, a producing species comprising one or more cells is incorporated into a screening unit when the producing species or a majority of the producing species is at a phase in its life cycle that is producing one or more of the compounds of interest.

In another embodiment of the invention, a producing species comprising one or more cells is incorporated into a screening unit when the producing species or a majority of the producing species is at a phase in its life cycle that produces a broad range of compounds.

Accordingly, a method of the invention for screening natural products, such as secondary metabolites, comprises the follwing steps in the order stated: coencapsulating a producing species and an assay system in a gel droplet to form a screening unit; placing the screening unit under conditions suitable for production of secondary metabolites for an interval sufficient for the secondary metabolites produced by the producing species to contact the assay system; and detecting a signal generated by the assay system.

An alternative method of the invention for screening secondary metabolites comprises the following steps in the order stated: encapsulating a producing species in a gel matrix to form a gel droplet; placing the gel droplet under conditions appropriate for growth and/or selection if the producing species contains recombinant or foreign DNA encoding a selectable marker; placing the gel droplet for an

interval under culture conditions such that production of secondary metabolites by the producing species begins; forming around the gel droplet (an outer layer of) a second gel matrix comprising an assay system to form a screening unit; placing the screening unit under the appropriate conditions for an interval sufficient for secondary metabolites produced by the producing species to contact the assay system; and detecting a signal generated by the assay system.

Alternatively, a producing species can be cultured until about the time when secondary metabolites production begins before coencapsulating the producing species and an assay system in a gel droplet to form a screening unit; placi ng the screening unit under conditions suitable for production of secondary metabolites for an interval sufficient for the secondary metabolites produced by the producing species to contact the assay system; and detecting a signal generated by the assay system.

The primary consideration with encapsulation of the producing species is to obtain production of natural products, including secondary metabolites. To improve natural product production, various modifications to fermentation conditions can be made. For example, the amount of available oxygen plays a significant role in the product of secondary metabolites. To increase the dissolved oxygen in the nutrient broth, silicone oil, perfluorocarbons, and hydrogen peroxide can be supplemented. Similarly, the use of baffled flasks, metal springs, and altering the volume to surface ratio of the culture can increase the availability of oxygen. Another variable is the use of different carbon sources, such as glucose, maltose, glycerol, etc. which can improve the production of natural products, such as secondary metabolites. The encapsulation conditions, such as the density of the alginate, can also play a major part in the production of various natural products. See, for example, (Roisin et al., Annals NY Acad Sci, 782:61-69). For optimal results, the production of natural products for each

producing species under various encapsulated conditions should be evaluated by techniques that are well known in the art such as chromatography, spectroscopy, etc. A producing species may also be induced to produce natural products by exposure to chemical inducers, or by manipulating environmental factors, such as nutrient concentration, oxygen concentration, temperature, accumulation of waste products, etc.

In one specific embodiment, the method of the invention comprises encapsulating a producing species in a gel droplet; placing the gel droplet under culture conditions for an interval such that production of natural products, such as secondary metabolites, begins; contacting an assay system with the gel droplet; and detecting a signal generated by the assay system. Gel droplets containing producing species may be used in a range of screening assay formats, including but not limited to conventional multiwell plates, vials and bacterial lawns. For example, gel droplets may be placed in a sterile grid or individually into the wells of a 96-well or 384-well microtitre plates and the plates are placed under culture conditions appropriate for growth and/or natural product production. At the appropriate time, assay system of the invention is added to the wells. After an interval sufficient for the natural product in the gel droplet to contact the assay system, signal generated by the assay system is detected or measured.

In various embodiments of the invention, where the producing species is a genetically engineered cell, the producing species is incorporated into a screening unit immediately following the introduction of recombinant or foreign DNA into the cell. The producing species may then be allowed to recover and grow for a period of time under conditions appropriate for growth and/or selection. For example, *Streptomyces* protoplasts may be allowed to regenerate while encapsulated, and cultured in the presence of antibiotics selection which will effect the retention of foreign DNA carrying a selectable marker. Producing species

containing recombinant or foreign DNA encoding a selectable marker can be selected while inside a screening unit by methods known in the art, such as the use of antibiotics or special nutritional conditions, etc. The selected producing species are subsequently placed under the appropriate conditions that facilitates production of compounds to an assay system.

In a specific embodiment of the invention where the producing species is a genetically engineered cell, in which the recombinant or foreign DNA is directly introduced from another cell (the donor) by methods known in the art, such as but not limited to conjugation, both the donor cell and the cell that will become the producing species (the recipient or transconjugants) can be coencapsulated in a gel droplet or screening unit. Conjugal transfer of DNA from one bacteria to another can be carried out within a gel matrix, such as calcium alginate (Steenson et al., 1987, Appl. Environ. Microbiol. 53(4):898-900). After conjugation, transconjugants carrying the transferred DNA can be selected, e.g., by antibiotics, or nutrient conditions, and cultured until secondary metabolites production begins while encapsulated in the gel droplet. As described above, the transconjugants in a gel droplet can be recovered for coencapsulation with an assay system, or further encapsulated with a second outer layer comprising an assay system.

In yet another embodiment, a producing species encapsulated within a gel droplet can be used for producing natural products, which are collected and concentrated by a coencapsulated solid carrier. Accordingly, a method of the invention for screening natural products, such as secondary metabolites, synthesized by a producing species comprises the following steps in the order stated: coencapsulating a producing species and a solid carrier in a gel droplet; placing the gel droplet under conditions suitable for production of secondary metabolites for an interval sufficient for the natural products produced by the producing species to sequester in the solid carrier; retrieving the

solid carrier from the gel droplet; contacting the solid carrier with an assay system under conditions such that the sequestered secondary metabolites contact the assay system; and detecting a signal generated by the assay system. If desired, the producing species may be permeabilized by an agent such that the natural products, including those not secreted by the producing species, can become sequestered in the solid carrier prior to dissolving the gel droplet and retrieving the solid carriers.

In yet another embodiment of the invention, where a producing species is a solid carrier loaded with one or more compounds, the producing species is encapsulated with an assay system in a screening unit and placed under the appropriate conditions that facilitates release and presentation of the compounds to the assay system. Accordingly, a method for screening chemical compounds, including natural products and secondary metabolites, comprises the following steps in the order stated: coencapsulating a producing species and an assay system in a gel droplet to form a screening unit, wherein the producing species is a solid carrier of a compound; placing the screening unit under conditions such that the compound contact the assay system; and detecting a signal generated by the assay system.

In yet another embodiment, the present invention provides a method for producing natural products comprising encapsulating a producing species in a gel droplet, incubating the gel droplet under conditions suitable for growth and/or production of natural products, such as secondary metabolites, wherein the gel droplet is not in contact with, or immersed or submerged in liquid. For example, the gel droplets can be placed on a sterile grid and incubated in an environment where the temperature and humidity can be controlled. This approach of dry microfermentation is an improvement over existing methods of producing natural products which is typically carried out in a fermentation reactor in which encapsulated cells are

submerged in liquid culture media. By not culturing the gel droplet in a liquid media, loss of natural products via diffusion of the natural products out of the gel droplet is reduced. For example, if a pool of cells from a gene expression library are encapsulated, each droplet serves as a microfermentation chamber where the individual producing species grow and mature to natural product production. A broad range of natural products can be produced by the individually encapsulated producing species and accumulated in the droplets without inadvertant mixing or loss. The natural products are concentrated within each individual gel droplet which can be independently assayed, identified, and retrieved by methods described above, for example, by coencapsulated with an assay system, and dissolving the droplet.

The various embodiments of the present invention is described in detail in the following subsections, and illustrated with working examples in Section 6.

5.1. ENCAPSULATION

A screening unit of the invention also referred to as a gel droplet, comprises a small volume of gel, in which are encapsulated or embedded the elements of a screening assay. A screening unit may comprise a single gel region, or a plurality of distinguishable gel regions. The gel regions can be of different gel matrix compositions, or of the same gel matrix compositions. Generally, the gel droplet is spherical in shape and has a diameter greater than one millimeter in diameter. The diameter range of the approximately spherical droplets is from about one millimeter to about ten millimeter, and preferably from about one millimeter to about three millimeter.

Any methods known in the art for forming small volumes of gel can be used to form the screening unit of the invention.

In various embodiments, a producing species or an assay system or both are encapsulated (or coencapsulated) in a gel

region as the gel region is being formed. Each gel region in a screening unit can comprise one or more producing species, and/or one or more assay system. A screening unit of the invention can be constructed with multiple gel regions, each formed separately and containing a different composition of producing species and/or assay systems. In one embodiment, both the producing species and the assay system(s) are coencapsulated in one gel region (See Figure 1A). In another embodiment, the gel regions of a screening unit are formed sequentially such that one gel region surrounds another gel region, thus, forming an outer layer and an inner layer (or inner core) (See Figure 1B.). The invention also provides that two or more independently formed gel regions can be coupled together by coencapsulating the gel regions to form a single screening unit. The process as described above may be repeated to form successive layers of gel regions each comprising a different composition of producing species and/or assay systems. As will be apparent to those skilled in the art, various configurations of gel regions comprising the desired composition of producing species and/or assay systems can be created to form a screening unit by permuting the composition in the gel matrix and the sequence of gel formation steps. This approach may be advantageously exploited for optimally positioning a producing species and an assay system relative to each other in a screening unit.

Gel droplets, can be made of by any number of gel matrices utilizing ionic or thermal gelling principles. Typically, producing species and assay systems are mixed with a fluid precursor of the gel matrix, which is then solidified by polymerization. A preferred gel matrix for the encapsulation of living cells should cause as little trauma to the cells as possible. There should, ideally, be no shock to the cells from change of temperature, change of osmotic pressure, change of chemical environment, and chemical reaction. Hydrogels are generally used because of high water retention and porosity range, which allows better diffusion of smaller solutes, such as nutrients and waste products.

Such matrices, many of which are known in the art, may include but are not limited to polysaccharides, such as alginates, carageenans, agarose, chitosan, cellulose, and pectins, and polyacrylamides.

Producing species and assay systems (including indicator cells) can be added to the gel matrix material to form a suspension and distributed evenly while the matrix is in liquid phase. Gel regions or gel droplets are formed by hardening of the matrix following the formation of small individual volumes of the gel matrix suspension droplets using any number of techniques. These techniques are well known with the art and may include, for example, emulsification with oil and high powered mixing, dropping using a syringe type apparatus, vibration of either a nozzle attached to a reservoir of the suspension or the suspension, atomizing, electrostatic forces, or a cutting method which uses rotating wires to cut the liquid droplets into small units. For a description in detail, see, for example, Jekel, M., and Vorlop K.-D., Bioencapsulation Technology, International Workshop on Bioencapsulation, 1996, T1, which is incorporated herein by reference in its entirety.

Gel matrices may also be modified in order to control the structure as well as the physical properties of the gel matrices, such as, the diffusion of gases and small molecules through the gel. Depending on the desired properties, the gel droplets of the invention may be appropriately modified by any methods known in the art, including but not limited to, the addition of a poly-lysine coating, or the addition of miscible organic solvent to impart a more nonpolar environment to one or more gel layers.

One of the preferred gel matrix is sodium alginate which is commercially available. Alginic acid is a copolymer of β-D-mannuronic acid and α-L-guluronic acid linked by (1->4)-glycosidic linkages, typically obtained from marine algae. For gel strength, high L-guluronate content is required. The formation of gels by interaction of alginate with divalent cations, such as Ca^{2+}, Sr^{2+}, and Ba^{2+} are well known in the art.

Uniform gels can be produced by arranging a uniform distribution of divalent ions throughout the fluid phase before setting. Calcium alginate gel is most preferred, and its use is described in the Working Examples in Section 6. When it is desired to free the contents of a calcium alginate gel droplet, this may be done by stirring the droplet with a suitable calcium chelating agent, such as EDTA, sodium citrate, or sodium hexametaphosphate. Methods for encapsulating cells in alginates are known and described in the art, for example, in Bucke, C., 1987, Methods Enzymol. 135:175-189, which is incorporated herein by reference in its entirety.

5.2. PRODUCING SPECIES

A producing species of the invention is the source of compounds in a screening unit. In one embodiment, the producing species is a traditional biological source of natural products, that includes any organism that has been used to produce a compound for the purpose of drug screening. Typically, it encompasses bacteria, fungal and plant cells, that are naturally occurring or selected by known biochemical, genetic or breeding techniques. Other sources of natural products with potentially useful antimicrobial or pharmacological properties that can be used include cells and cell lines of invertebrates and vertebrates.

Environmental samples obtained from natural or man-made environments, can also be used as producing species in the methods of the invention. Generally, microorganisms growing under stressed or competitive environments are rich in secondary metabolites, and are thus attractive sources of useful compounds. Typically, such samples contain a mixture of organisms, some of which may be unidentified. For the purpose of an initial drug screen, the producing species need not be taxonomically defined or biochemically characterized. Samples can either be randomly collected or collected from areas that are ecologically stressed, for example, near an industrial effluent, or areas that are biologically

competitive, for example, rich soil, or rhizosphere. Soil, freshwater or seawater filtrates, deposits around hot springs or thermal vents, and marine or estuarine sediments may be used as sources of producing species. Samples may be collected from benthic, pelagic, and intertidal marine sources. Samples may be collected from tropical, subtropical, temperate and other regions. The producing species may be thermophilic, halophilic, acidophilic, barophilic, or methanogenic organisms.

Accordingly, the producing species contemplated by the invention may include, but are not limited to viruses; bacteria; unicellular eukaryotes, such as yeasts, algae and protozoans; multicellular eukaryotes, invertebrate cells and vertebrate cells, such as but not limited to algae, fungi, plants, helminths, parasites, tunicates, bryozoans, worms, echinoderms, insects, mollusks, fishes, amphibians, reptiles, birds, and mammals. In some aspects, whole live organisms of the appropriate dimensions may be used as the producing species. In many aspects, for ease of operation, it is desirable that the producing species is unicellular or can be cultured and manipulated as single cells. Spores, mycelial fragments, protoplasts, and other forms of a producing species may also be encapsulated. Non-limiting examples of producing species are listed in Tables I and II.

Table I:

List of exemplary bacterial and fungal producing species (Berdy 1974, Adv Appl Microbiol, 18: 309-406; Goodfellow et al. 1989, in "Microbial Products: New Approaches", Cambridge University Press 343-383)

Group		Genera
Bacteria		
	Actinomycetales	*Streptomyces, Micromonospora, Norcadia, Actinomadura, Actinoplanes, Streptosporangium, Microbispora, Kitasatosporia*
	Eubacteriales	*Azobacterium, Rhizobium, Achromobacterium, Enterobacterium, Brucella, Micrococcus, Lactobacillus, Bacillus, Clostridium, Brevibacterium*
	Pseudomonadales	*Pseudomonas, Aerobacter, Vibrio, Halobacterium*
	Mycoplasmatales	*Mycoplasma*
	Myxobacteriales	*Cytophaga, Myxococcus*
Fungi		
	Myxothallophytes	*Physarum, Fuligo*
	Phycomycetes	*Mucor, Phytophtora, Rhizopus*
	Ascomycetes	*Aspergillus, Penicillium*
	Basidiomycetes	*Coprinus, Phanerochaete*
	Fungi Imperfecti	*Acremonium (Cephalosporium), Trochoderma, Helminthosporium, Fusarium, Alternaria, Myrothecium*
	Yeasts	*Saccharomyces*

Preferred species of *Streptomyces* may include but are not limited to *Streptomyces lividans, Streptomyces coelicolor, Streptomyces fradiae, Streptomyces venezuelae, Streptomyces roseosporus, Streptomyces toyocaenesis, Streptomyces griseus, Streptomyces clavuligerus, Streptomyces lavendulae, or Streptomyces arenae.*

Table II:

Higher forms of exemplary producing species

Group	Exemplary Genera, Compounds & Properties
Plants	
Algae	*Digenea simplex* (kainic acid, antihelminthic) *Laminaria anqustata* (laminine, hypotensive)
Lichens	*Usnea fasciata* (vulpinicacid, antimicrobial; usnic acid, antitumor)
Higher Plants	*Catharanthus* (Vinca alkaloids), *Digitalis* (cardiac glycosides), *Podophyllum* (podophyllotoxin), *Taxus* (taxol), *Cephalotaxus* (homoharringtonine), *Camptotheca* (Camptothecin), *Artemisia* (artemisinin), *Coleus* (forskolin), *Desmodium* (K channel agonist)
Protozoa	
Dinoflagellates	*Ptychodiscus brevis* (brevitoxin, cardiovascular)
Insects	*Dolomedes* ("fishing spider" venoms), *Epilachna* (mexican bean beetle alkaloids)
Bryozoans	*Bugula neritina* (bryostatins, anti cancer)
Molluscs	*Conus toxins*
Sponges	*Microciona prolifera* (ectyonin, antimicrobial) *Cryptotethya cryta* (D-arabino furanosides)
Corals	*Pseudoterogonia species* (Pseudoteracins, anti-inflammatory) *Erythropodium* (erythrolides, anti-inflammatory)
Worms	
Annelida	*Lumbriconereis heteropa* (nereistoxin, insecticidal)
Spinunculida	*Bonellia viridis* (bonellin, neuroactive)
Tunicates	*Trididemnum solidum* (didemnin, anti-tumor and anti-viral) *Ecteinascidia turbinata* (ecteinascidins, anti-tumor)
Fish	*Eptatretus stoutii* (eptatretin, cardioactive), *Trachinus draco* (proteinaceous toxins, reduce blood pressure, respiration and reduce heart rate)

Amphibians	*Dendrobatid frogs* (batrachotoxins, pumiliotoxins, histrionicotoxins, and other polyamines)
Reptiles	Snake venom toxins
Birds	histrionicotoxins, modified carotenoids, retinoids and steroids (Goodwin 1984 in "The Biochemistry of the Carotenoids" Vol. II, Chapman and Hall, New York, pp. 160-168)
Mammals	*Orinthorhynohus anatinus* (duck-billed platypus venom), modified cantenoids, retinoids and steroids (Goodwin 1984, supra, pp. 173-185; Devlin 1982 in "Textbook of Biochemistry", Wiley, New York, p. 750)

The present invention is not limited to the use of naturally occurring organisms as producing species. The producing species can also be a genetically manipulated organisms or cells, which include recombinant prokaryotic cells, or recombinant eukaryotic cells (including cells derived from a transgenic organism), that contain heterologous or foreign genetic material. Thus, any genetically engineered prokaryotic and eukaryotic cells of the organisms listed in Tables I and II can also be used as a producing species.

In various embodiments of the invention genetically engineered cells from an expression library known to or suspected to produce a range of unidentified compounds may be used as producing species. Alternatively, cells that are genetically engineered to overproduce and/or to secrete a class of compounds can also be used. Foreign genetic material can be introduced into the organisms by techniques known in the art, such as those described in Section 5.3.1, including but not limited to transformation, transfection, transduction, conjugation, electroporation, protoplast fusion, cell fusion, and liposome-mediated methods.

The methods of the invention can be advantageously used to screen gene expression libraries in microorganisms built for the purpose of generating novel biosynthetic pathways by combining the genetic materials derived from a plurality of organisms. These pathways require multiple proteins (specifically, enzymes) to synthesize the compounds of interest, entailing greater complexity than expressing single proteins as potential drug candidates.

For example, a host-vector system in *Streptomyces* has been developed that allows directed mutation and expression of polyketide synthase (PKS) genes (McDaniel et al. 1993, Science 262:1546-1550; Kao et al. 1994, Science 265:509-512). Since the PKSs catalyze multiple steps of the biosynthesis of polyketides and control the structural diversity of the polyketides produced, this host-vector system has been used to develop more efficient ways of producing polyketides, and

to rationally develop novel polyketides (Khosla et al., WO 95/08548). Accordingly, recombinant *Streptomyces* host cells producing potentially novel polyketides can be used as producing species of the invention to identify novel antibiotics against specific classes of microorganisms, and other pharmacological or immunomodulating activities.

In a specific embodiment, the methods of the invention can be used with combinatorial gene expression libraries to identify potentially useful medically-active metabolites, agricultural chemcials, and specialty chemicals. Generally, recombinant host cells in these combinatorial gene expression libraries comprise a random assortment of genetic material derived from the genomes of a plurality of organisms. The mixture of genetic material used for constructing combinatorial gene expression libraries may be preselected by hybridization with known functional genes, such that the cloned genetic material comprises largely related genes encoding partial or complete biosynthetic pathways of different organisms (e.g., pathways for synthesis of polyketides, aminoglycosides, nucleosides, non-ribosomal peptides, β-lactam, terpene, shikimate-derived natural products). In these combinational gene expression libraries, novel combinations of genetic material from different organisms, which for the most part do not occur in nature, is expressed in recombinant library cells to produce functional proteins, that in turn generate metabolites of interest. For some libraries, using the appropriate host-vector system, the cloned genetic material can be transferred from one species of host organism (the donor) to another species or strain of host organism (the recipient) by for example, conjugation. The transferable genetic material in these mobilizable combinatorial gene expression libraries can be stably maintained and expressed in different recipients. Recombinant cells in combinatorial gene expression libraries are highly suitable for use as producing species of the invention for high throughput drug screening since the cells in such libraries are all of the same type and can be

encapsulated in the same manner with an assay system; each encapsulated library cell is potentially capable of producing a novel and unique compound for screening.

In a specific embodiment of the invention, the process of conjugation, which facilitates DNA transfer between two compatible bacterial species or strains, can be conducted within a gel droplet. In this instance, the donor and the recipient are coencapsulated within a gel droplet wherein conjugal transfer take place through the gel matrix. Moreover, after the transfer, recipients containing the transferred genetic material can be selected and grown while they remain encapsulated.

Methods for making and using combinatorial gene expression libraries are described in WO 96/34112, published October 31, 1996, and in a copending United States patent application, serial number 08/738,944, both of which are incorporated herein by reference in their entireties.

In another embodiment, a producing species of the invention can be a solid carrier which contains potential drug candidates to be presented to an assay system. Compounds to be added to a solid carrier can be obtained from a wide variety of sources including collections of natural products in the form of bacterial, fungal cells and plant and animal extracts; and synthetical chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide variety of chemical structures. In addition, natural products or known antibiotic compounds may be subjected to random or directed chemical modifications to produce derivatives and structural analogs that can be added to the solid carrier of the invention.

Any methods known in the art can be used to add a compound of interest to the solid carrier, including but not limited to adsorption, covalent linkages, etc. In a specific embodiment, a compound may be chemically coupled or crosslinked to the solid carrier via a cleavable linker, such that the coupled or crosslinked compound can be released by

cleaving the linker using the appropriate physical or chemical method.

By "solid carrier" is intended any support capable of binding a compound. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either hydrophilic or hydrophobic. The support material may have virtually any possible structural configuration so long as the coupled compound is capable of contacting an assay system or can be released by the appropriate method. Thus, the configuration may be spherical, as in a bead. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding or coupling compounds, or will be able to ascertain the same by use of routine experimentation. Typically, the solid carrier comprises a covalently bonded linker with a functional group, such as a primary amine or carboxylic acid group, at one end of the linker, which is available for the coupling reaction. For example, commercially available solid carriers commonly used for combinatorial chemical synthesis may be used in this embodiment of the invention, for example, Hydroxymethyl-Photolinker NovaSyn® (Affymax Research Institute).

It is well known in the art that resins with photocleavable attachment sites can be used to build libraries of synthetic compounds via combinatorial chemistry. Chemical libraries bound to resins can be screened by encapsulation in a solid carrier, such as an alginate droplet, by methods of the invention. These droplets can be put on agar plates and irradiated with light of an appropriate wavelength in order to photochemically cleave the synthetic compounds from the resin. The compounds will diffuse out of the alginate and onto the agar which can be overlayed with an assay system to determine the biological activity of the compound(s) in the library. The conditions of illumination may be adjusted so that the compounds are slowly cleaved from the resin to provide a diffusion gradient

of constant concentration rather than a pulse. The illumination can also be adjusted to leave some amount of synthetic compound on the resin, so that the biologically active compound can be identified later. For example, if the illumination of the droplets is unidirectional, half of the resin will be shielded from photocleavage and still contain bound synthetic compounds. Thus, one can identify the compounds that gave a positive result by retrieving the resin from alginate bead, and releasing the compound from the resin for analysis by mass spectrometry or other physical means.

In another specific embodiment, to facilitate detection of compounds of interest produced by a producing species, absorptive materials such as neutral resins, e.g., Diaion HP20 or Amberlite XAD-8 resin in the form of small beads, may be coencapsulated with a producing species in a gel region of a screening unit or encapsulated in a neighboring gel region of the screening unit, such that secondary metabolites can come into contact with the neutral resin. Since many secondary metabolites are hydrophobic molecules, the release or secretion of such metabolites by a producing species may lead to precipitation on the cell exterior. Inclusion of such resins as solid carriers of metabolites in the culture causes sequestration of the secondary metabolites to occur on the carrier which may be removed from the culture for elution, or for use directly in a screening assay. Subsequent to loading the solid carriers, such carriers may be encapsulated in a separate screening unit as a producing species that can present the sequestered compounds to an assay system. The presentation of compounds to an assay system by the encapsulated carriers may be induced in a screening unit by using physical or chemical technologies which cause carrier the release of the metabolites from the carrier.

5.3. ASSAY SYSTEMS

An assay system as provided by the present invention is a means for detecting a desired activity which is

encapsulated within a gel droplet or screening unit. An assay system directly or indirectly produces a detectable and/or measurable signal when it contacts or reacts with a compound having the desired activity.

The methods of the invention provide alternative but not mutually exclusive approaches to assay for a desired activity thereby detecting and isolating the producing species of interest. Depending on the desired activity, which includes but is not limited to antibiotic, antiviral, antifungal, antiparasitic, pesticide, phamacologcial or immunological activity, an assay system may comprise chemical indicators, indicator cells, reporter molecules, and reporter regimens.

5.3.1. INDICATOR CELLS

In one embodiment of the invention, indicator cells or organisms are used as the assay system for signaling the production of a desirable activity or compound, thereby enabling identification and/or isolation of the producing species.

Indicator cells may be co-encapsulated with a producing species to form a screening unit. Alternatively, indicator cells may be encapsulated in one gel region in a screening unit, wherein compounds produced or presented by a producing species in another gel region can diffuse into the gel region containing the indicator cells.

Indicator cells are selected for their biological properties which is responsive to the presence of the desirable activity or compound. The term indicator cells as used herein refers to whole live or fixed cells, organelles, cell lysate, or nuclear or cellular fractions thereof. Other forms of an indicator organisms that can be used include but is not limited to spores, mycelial fragments, and protoplasts.

Indicator cells may also be the targets of the desirable compound in which case, the decrease or lack of growth of the indicator cells, or the death of the indicator cells signals the presence of a desired activity. The detection and/or

enumeration of viable or dead cells can be facilitated by stains, dyes or other techniques known in the art.

Indicator cells used in this invention for purpose of identifying antibiotics (and antifungal compounds) may include pathogenic and non-pathogenic microorganisms for the detection of antimicrobial or antifungal activity. Suitable indicator cells may include but are not limited to *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Microsporum canis*, *Micrococcus luteus*, *Botrytis cinerea*, *Phytophthora infestans*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum*.

Indicator cells may also include animal cells for the detection of compounds that cause cytotoxicity, cell proliferation, apoptosis, and/or other changes in cellular phenotype. For example, such indicator cells may include any neoplastic cells or tissues isolated from a plant or an animal, including a human. Any cell derived from an animal infected by a pathogen, in particular, an intracellular pathogen, such as a virus, a bacterium, fungus, parasite or protozoan, can also be used as an indicator cell. Primary cell cultures, tissue explants, and permanent cell lines derived from cancer tissues, cancer cells, or infected cells may also be used in the invention.

Indicator cells may also include whole live organisms including protozoa and helminth parasites such as nematodes, e.g. *Caenorhabditis elegans*. Insects at various stages of development (e.g., *Drosophila* embryos) or various phases of their life cycles (e.g., larva) may be used as indicator cells. For example, eggs and larvae of insects which are agricultural pests, or hazards to human and/or animal health can be used as indicator cells to screen for insecticides and pesticides.

In another embodiment of the invention, the indicator cell is genetically engineered to produce a target molecule of the desired compound(s). Standard recombinant DNA techniques may be applied to introduce a construct containing sequences encoding the target molecule (i.e., the target

gene), and to obtain expression of the target molecule in the indicator cells. The target gene is operably linked to a promoter in the reporter cell to produce receptor transcripts which may be processed and translated into a functional molecule.

Described herein are methods for making an indicator cell using a target gene, which are also applicable to the making of a reporter cell using a reporter gene as described in Section 5.3.2.

In the present invention, the target gene sequence(s) may be inserted into a recombinant expression vector. The term "target gene constructs" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of target gene sequences. Such target gene constructs of the invention are preferably plasmids which contain a promoter sequence which is operably associated with the inserted target gene sequence. It typically contains an origin of replication as well as specific genes which allow phenotypic selection of the transformed cells.

"Operably-associated" or "operably-linked" refers to an association in which the promoter and the target gene sequence are joined and positioned in such a way as to permit transcription. Two or more sequences, such as a promoter and any other nucleic acid sequences are operably-associated if transcription commencing in the promoter will produce an RNA transcript of the operably-associated sequences.

A target gene construct useful in the invention may also contain selectable or screenable marker genes for initially isolating, identifying or tracking test cells that contain heterologous DNA. The target gene construct may also provide unique or conveniently located restriction sites to allow severing and/or rearranging portions of the DNA inserts in a target gene construct. More than one target gene may be inserted into the construct such that the indicator cells containing the resulting construct can be assayed by different means.

Transformation of the host cell with the recombinant DNA may be carried out by conventional techniques well known to those skilled in the art. For example, where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl could be used.

In addition to conventional chemical methods of transformation, the plasmid vectors of the invention may be introduced into an indicator cell by physical means, such as by electroporation or microinjection. Electroporation allows transfer of the vector by high voltage electric impulse, which creates pores in the plasma membrane of the indicator cell and is performed according to methods well known in the art. Additionally, cloned DNA can be introduced into indicator cells by protoplast fusion, using methods well known in the art.

In cases where plant expression vectors are used, these constructs can be introduced into plant indicator cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques *see*, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

In mammalian indicator cells, a variety of commercially available mammalian expression vectors can be used. In addition, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the donor DNA sequence can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3)

will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts. (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Viral vectors based on retroviruses can also be used (Morgenstern et al. 1989, Ann Rev Neurosci, 12:47-65). Alternatively, the vaccinia 7.5K promoter can be used. (See, e.g., Mackett et al. 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al. 1984, J. Virol. 49:857-864; Panicali et al. 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

For long-term expression of a target molecule in an indicator cell which will be used in a large scale screening project, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered indicator cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the introduced DNA confers resistance to the selection and allows cells to stably integrate the target gene sequence into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For stable expression of the target gene, a number of selection systems can be used for mammalian cells, including but not limited to the Herpes simplex virus thymidine kinase (Wigler, et al. 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al. 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (dhfr), which confers resistance to methotrexate (Wigler, et al. 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al. 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers

resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al. 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre, et al. 1984, Gene 30:147).

The indicator cells which contain the target gene sequence and which express the target gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, etc.); (c) assessing the level of transcription as measured by the expression of target mRNA transcripts in the host cell; and (d) detection of the target gene product as measured by immunoassay or by its biological activity.

The target molecule to be expressed in an indicator cell may be an intracellular protein, a cell surface protein, or a secreted protein. The target molecule may also be a protein, that is normally located intracellularly, but has been modified by techniques known in the art so that it resides on the cell surface, or it is secreted. Non-limiting examples of target molecules include receptors for hormones, cytokines, neurotransmitters, adhesion molecules, oncogenes, transcription factors, signaling molecules, such as kinases and phosphatases, etc.

Interaction of the target molecule and the desired compound within the screening unit may produce a detectable signal. Interaction of the target molecule with the desired compound(s) on the cell surface or inside the indicator cell may initiate or modulate biological signal transduction. As used herein, the term signal transduction is not limited to transmembrane signaling, but includes the various signaling pathways that branch throughout the cell and into the nucleus of eukaryotic cells, including mammalian and human cells.

Desired compound(s) produced by the producing species are detected by detecting or measuring changes in cellular phenotype or cellular processes that are affected by signal transduction in the indicator cells. Such cellular processes may include, but are not limited to transcription of specific gene(s), division, growth, differentiation, adhesion, apoptosis, as well as abnormal cellular processes, such as but not limited to, cell fusion, dedifferentiation, blocking of differentiation, transformation, and metastasis. Any standard techniques may be used to detect, observe, and measure such changes in cellular phenotype or cellular processes. Alternatively, indicator cells may be used in conjunction with a reporter regimen to generate a detectable signal. Components of a reporter regimen may be encapsulated in a neighboring gel region or coencapsulated in the same region.

5.3.2. INDICATOR CELLS COMPRISING A REPORTER GENE

In another embodiment of the invention, the assay system comprises an indicator cell that is genetically engineered to contain a reporter gene construct ("a reporter gene").

The term "reporter gene" as used herein refers to any genetic sequence that is detectable and distinguishable from other genetic sequences present in an indicator cell. The reporter gene sequence encodes a protein that is readily detectable either by its presence, or by its activity that results in the generation of a detectable signal. A reporter gene is used in the invention to monitor and report the presence of a desired activity in a gel droplet.

In various embodiments of the invention, a reporter gene is operably linked to a promoter in the reporter cell to produce receptor transcripts which may be processed and translated into a functional reporter molecule. Many reporter genes have been described, and some are commercially available for the study of gene regulation in a large variety of organisms. See, for example, Alam and Cook, 1990, Anal.

Biochem. 188:245-254, the disclosure of which is incorporated herein by reference.

Any antigenic peptide or protein that can be detected by an antibody can be used as a reporter, for example, growth hormone (Selden et al., Mol. Cell Biol., 6:3173). To facilitate detection by antibody binding in immunoassays, antigenic reporter molecules that are secreted or attached on the test cell surface are preferred.

For convenience and efficiency, enzymatic reporters and light-emitting reporters are preferred for the methods of the invention. Accordingly, the assay systems of the invention encompasses principles of histochemical, colorimetric, fluorometric and chemiluminescent assays.

A variety of enzymes may be used as a reporter which includes but are not limited to β-galactosidase (Nolan et al. 1988, Proc Natl Acad Sci USA 85:2603-2607), chloramphenicol acetyltransferase (CAT; Gorman et al., 1982, Mol Cell Biol, 2:1044; Prost et al., 1986, Gene 45:107-111), β-lactamase, β-glucuronidase and alkaline phosphatase (Berger et al., 1988, Gene 66:1-10; Cullen et al., 1992, Methods in Enzymol, 216:362-368). Transcription of the reporter gene leads to production of the enzyme in the reporter cell. The amount of enzyme present can be measured via its enzymatic action on a substrate resulting in the formation of a detectable reaction product in a screening unit. An enzyme that has found extensive use as a reporter in prokaryotic and eukaryotic cells is bacterial β-galactosidase, especially *E. coli* β-galactosidase. The enzyme is very stable and has a broad specificity so as to allow the use of different substrates, such as but not limited to 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and fluorescein galactopyranoside (Molecular Probes, Orgeon). See, Nolan et al. 1988, Proc Natl Acad Sci USA 85:2603-2607. Another commonly used reporter gene is the *E. coli* β-glucuronidase gene (GUS; Gallagher, 1992, in "GUS protocols", Academic Press) which can be used with various histochemical and fluorogenic substrates, such as X-glucuronide, and 4-methylumbelliferyl

glucuronide. See, also for example, U.S. Patent No. 5,070,012, and WO 96/30540.

A variety of bioluminescent, chemiluminescent and fluorescent proteins can also be used as light-emitting reporters in the invention. One type of such reporters, which are enzymes and require cofactor(s) to emit light, include but are not limited to, the bacterial luciferase (luxAB gene product) of *Vibrio harveyi* (Karp, 1989, Biochim Biophys Acta 1007:84-90; Stewart et al. 1992, J Gen Microbiol, 138:1289-1300), and the luciferase from firefly, *Photinus pyralis* (De Wet et al. 1987, Mol Cell Biol 7:725-737).

Another type of light-emitting reporter, which does not require substrates or cofactors, includes but are not limited to the wild type green fluorescent protein (GFP) of *Victoria aequoria* (Chalfie et al. 1994, Science 263:802-805), and modified GFPs (Heim et al. 1995, Nature 373:663-4; PCT publication WO 96/23810). Transcription and translation of this type of reporter gene leads to accumulation of the fluorescent protein in the reporter cells, which can be detected by photography and/or measured by using a fluorimeter. Methods for detecting fluorescence and performing assays on fluorescent materials are well known in the art and are described in, e.g., Lackowicz, J.R., 1983, Principles of Fluorescence Spectroscopy, New York: Plenum Press.

Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, e.g., substrates and cofactors for reporter molecules that are enzymes. Such compositions are well known in the art. Components of a reporter regimen may be encapsulated in a gel region, or coencapsulated with the reporter cell such that it is

available when the compounds of interest are present in the screening unit.

For example, a human cancer cell line is transfected or transformed with a reporter construct comprising the gene encoding green fluorescent protein operably-associated with a constitutive promoter, such as the immediate early promoter of human cytomegalovirus (CMV). Recombinant cancer cells containing the reporter construct are coencapsulated with a producing species in a screening unit, and placed under culture conditions that allow cancer cell growth. Recombinant cells containing the GFP reporter construct may be encapsulated in a gel matrix, such as sodium alginate, and the fluorescence signal is detectable by inspection under a fluorescence microscope, or other standard techniques such as digital imaging or fluorescence activated cell sorter (FACS). A reduction of fluorescence intensity in a screening unit relative to other screening units containing growing cancer cells may indicate that compound(s) which causes cancer cell death or inhibition of cancer cell growth is present.

For example, an inducible promoter system for the detection of chemicals which cause an SOS response in an *E. coli* target cell can be used (Little, J.S. and Mount, D.W. The SOS Regulatory System of *Escherichia coli*, Cell, 29, 1982, 11-22). Upon exposure to DNA-damaging agents which cause cleavage of a repressor to promoters such as sfiA or dinD, production of the promoter is induced in the effected cell. This activity can then be detected colorimetrically using a substrate such as X-gal or fluorescently using FDG (Molecular Probes, Eugene, Oregon).

In a specific embodiment, a reporter cell can be genetically engineered to produce a target molecule of the desired compound(s), and a reporter gene operably-associated with a promoter that responds directly or indirectly to the interaction of the target molecule with the desired compound(s). Promoters that may be used to make reporter construct are those naturally associated with genes encoding the end product of a signaling pathway, or genes encoding

signaling molecules that are downstream of the target molecule in a signaling pathway. The screening unit producing the desired compound(s) is identified by detectable signals generated by the induced production of the reporter molecule. Any combination of target molecule, reporter gene and reporter regimen known in the art and as described above may be used in this embodiment of the invention.

5.3.3. CHEMICAL INDICATORS

In an alternative embodiment, a chemical indicator or physiological probe can be used which generates a signal in response to a chemical or physiological change in a screening unit as the result of the presence of a desirable activity or compound. Such a probe or chemical indicator may be a precursor of a reporter molecule that is converted directly or indirectly to the reporter molecule by an activity or a compound sought in the screening process. Upon contact with a desired activity or compound, the chemical indicator or physiological probe generates a detectable signal which enables identification and/or isolation of the screening unit containing the productive species. Contact may be effected by coencapsulation of the chemical indicator and the producing species. Alternatively, contact may be effected by encapsulation of the chemical indicator in one gel region and diffusion of the indicator to another gel region comprising the producing species.

5.4. METHODS OF THE INVENTION

The invention may be better understood by reference to the specific embodiments described below which are illustrated by working examples set forth in Section 6.

5.4.1. COENCAPSULATION OF *ACTINOMYCETES* WITH INDICATOR CELLS

Alginate is chosen for the encapsulation of *Actinomycetes* species, because of its high permeability which improves accessibility to nutrients and oxygen, and removal

of waste products, and its mechanical strength which provides a semisolid substrate to which the bacteria adhere. Under optimal culture conditions, encapsulated *Actinomycetes* generally require at least two days to begin secondary metabolite production. One group of *Actinomycetes* that is particularly useful are the *Streptomyces*, including all naturally occurring wild type and mutant strain as well as genetically selected and genetically engineered strains. The assay system is an indicator cell, which is the target of a desired chemical produced by the *Actinomycetes* species, and may include microbial cells, mammalian cells, or whole organisms. Depending on the biology of the *Actinomycetes* species and the nature of the assay, three strategies of encapsulation are available for forming a screening unit of the invention.

The first strategy comprises coencapsulating the producing species preferably an *Actinomycete* and the indicator cells in a single gel droplet, and incubating the droplet under conditions suitable for secondary metabolite production, such that secondary metabolites are produced by the *Actinomycete* and that the metabolites contact the indicator cells in the droplet.

The second strategy comprises incubating the producing species preferably an *Actinomycete* in liquid culture media until about the time when secondary metabolite production begins, and coencapsulating the *Actinomycete*, wherein the *Actinomycete* with the indicator cells in a single gel droplet has grown into individual mycelial colonies (or "mycelial balls") and are producing secondary metabolites. The secondary metabolites produced by the *Actinomycete* diffuse throughout the droplet and come into contact with the coencapsulated indicator cells.

The third strategy comprises the initial encapsulation of the producing species, preferably an *Actinomycete*, by itself in a first gel region until about the time when secondary metabolite production begins. The *Actinomycete* can be encapsulated in the form of spores, cells, or mycelia,

mycelial fragments, or in the case of genetically engineered *Actinomycete,* also in the form of transformed protoplasts or transconjugants. Following secondary metabolite production by the *Actinomycete* in the first gel region, a second gel region of alginate containing indicator cells are formed on the outside of the first gel region, such that secondary metabolites can diffuse into the second gel region where they contact the indicator cells. Alternatively, the gel droplets containing encapsulated *Actinomycete* that are producing secondary metabolites can be directly used in a screening assay where the secondary metabolites could contact the indicator cells. Typically, without limitation, such a screening assay is carried out in microtitre plates, vials, or designated areas on a petri dish.

In these strategies, the critical considerations are the production of secondary metabolites, and the timing and spatial positioning of the indicator cells near the producing *Actinomycetes* species in order to obtain a biological effect of the secondary metabolites on the indicator cells.

5.4.2. COENCAPSULATION OF PRODUCING SPECIES WITH INDICATOR CELLS

Alginate is also suitable for the encapsulation of most natural product producers, such as bacteria, fungal cells, plant cells, and the like, because of its high permeability to nutrients, oxygen, and waste products, and its mechanical strength which could withstand the growth of the encapsulated producing species, and culture conditions which may involve vigorous agitation.

According to the invention, natural product producers such as bacteria, fungi, plants and the like, can be assayed for antimicrobial or antifungal chemicals by coencapsulating with microbial indicator cells. Generally, the producing species, in this case, a natural product producer, is encapsulated in a gel region, and cultured under the appropriate conditions until about the time when the desired

natural products and/or related compounds (usually secondary metabolites) production begins. Any methods known in the art can be applied to determine the time when production of the desired natural products begins in the producing species, including, for example, taking samples of gel droplets in culture at various time points, retrieving the producing species from the gel droplets, and assaying for the presence of the desired natural products and/or related secondary metabolites. When natural products or secondary metabolites production begins in the encapsulated producing species, a second gel region of alginate containing the indicator cells are formed on the outside of the first gel region, such that the natural products or related compounds can diffuse into the second gel region where they contact the indicator cells. Alternatively, the gel droplets containing encapsulated producing species that are producing secondary metabolites can be directly used in a screening assay where the secondary metabolites could contact the indicator cells. Typically, without limitation, such a screening assay is carried out in microtitre plates, vials, or designated areas on a petri dish. For the screening of antibiotics or antifungal compounds, the lack of or decreased growth of the indicator cells relative to a control cell (i.e, cells not contacted with any natural products) indicates that the encapsulated or coencapsulated producing species is producing at least one natural product, protein or compound of interest.

The primary consideration is the production of the natural products, and the timing and spatial positioning of the indicator cells near the producing species in order to obtain a biological effect of the natural products on the indicator cells.

6. EXAMPLE

The following subsections describe the screening of *Streptomyces* species for production of antibiotics, and a DNA-damaging agent, mitomycin C. The screening can be carried out using a number of possible encapsulation methods.

The successful detection of antibiotics production depends on the temporal and nutrient requirements of *Streptomyces* for exhibiting secondary metabolite synthesis, as well as the spatial positioning of the target species near the producing species in a screening unit.

6.1. COENCAPSULATION OF *STREPTOMYCES* AND E. COLI CONTAINING A REPORTER CONSTRUCT

Two types of *Streptomyces* stocks (spore suspension and mycelial pellets) were prepared using *Streptomyces lavendulae* and *Streptomyces lavendulae* 11E.3 (a non-mitomycin C producing mutant). The spore suspensions were harvested from a nutrient (F10A) agar plate that had been incubated for approximately 14 days at 30°C. These suspensions were filtered through sterile cotton to remove mycelial fragments. The mycelial pellets were then made by inoculating 50 μl cultures of trypticase soy broth (TSB) in 250 ml baffled flasks with 50 μl of the corresponding spore suspension. The liquid cultures were then incubated at 30°C for 36 hours while shaking at 400 rpm. After 36 hours, the mycelial cultures were centrifuged in 50 ml conical tubes for 45 minutes at 15,000 rpm and rinsed four times with 10% sucrose. The pellets were then stored at 4°C.

A sodium alginate solution (Protanal HF 120, Prenova Biopolymer a.s., Drammen, Norway) was prepared by dissolving sodium alginate powder at a concentration of 1% in a mixture of 50% sterile water and 50% LB media, using an overhead mixer at 2000 rpm. To this solution was added 10,000 cells per ml of a synchronized culture of *E. coli* containing a dinD DNA-inducible promoter system. In the presence of a DNA damaging agent, the SOS response in *E. coli* causes the cleaving of a repressor which results in the induction of the production of the enzyme β-galactosidase. This alginate mixture was then split in two and each received 1 μl per ml of a mycelial stock of either *S. lavendulae* or the mutant *S. lavendulae* 11E.3. These mixtures were allowed to degas and 10 ml were then extruded through a syringe with a 25

gauge needle into 250 ml baffled flasks containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off and replaced with 50 ml of nutrient media (ATCC sporulation media #5 with only 0.2% glucose - SM5A) with 5 μg/ml tetracycline. The coencapsulated *E. coli* and *S. lavendulae* were then incubated at 30°C while shaking at 400 rpm for three days. Every 24 hours the media was removed and fresh media was added.

On the third day the media was changed and 50 μl of X-gal at 40 mg/ml in DMSO was added to each flask. The cultures were then incubated for two hours at 37°C while shaking at 400 rpm. After one hour the gel droplets were examined under a dissecting microscope and the *E. coli* colonies from the *S. lavendulae* condition were beginning to turn blue, indicating the presence of β-galactosidase, while those in the mutant *S. lavendulae* 11 E.3 did not turn blue. After two hours, the colonies in the *S. lavendulae* condition were a much stronger blue color, indicating the induction of the promoter due to the presence of a DNA-damaging agent (mitomycin C), while the mutant condition showed no sign of β-galactosidase induction.

6.2. COENCAPSULATION OF *STREPTOMYCES* FILAMENTOUS COLONIES AND *E. COLI* CONTAINING A REPORTER CONSTRUCT

Liquid cultures of *S. lavendulae* and the mutant *S. lavendulae* 11E.3 were inoculated with 50 ml of a spore suspension and grown in 50 ml of ATCC sporulation media #5 (SM5) in 250 ml baffled flasks for two days at 30°C with shaking at 400 rpm. After two days of growth in liquid, *S. lavendulae* forms filamentous colonies ("mycelial balls") and has begun secondary metabolite production of mitomycin C. At this time, with the production of mitomycin C, the filamentous colonies are coencapsulated with an *E. coli* strain containing a dinD DNA-inducible promoter system.

A 1 % sodium alginate solution was prepared in a mixture of 50% sterile water and 50% LB media. To this solution were added 10,000 cells per ml of a synchronized culture of an *E. coli* strain containing a dinD DNA-inducible promoter system. This mixture was then split in two and each received 125 µl per ml alginate of the culture of either *S. lavendulae* or the mutant *S. lavendulae* 11E.3 filamentous colonies. These mixtures were then allowed to degas and 10 ml of each was extruded through a syringe with a 20 gauge needle into 250 ml baffled flasks containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off and replaced with 50 ml of nutrient media (SMSA) with 5 µg/ml tetracycline. The coencapsulated *E. coli* and *S. lavendulae* were then incubated at 30°C while shaking at 400 rpm for three days. Every 24 hours this media was removed and fresh media was added.

On the third day the media was changed and 50 µL of X-gal at 40 mg/ml in DMSO was added to each flask. The cultures were then Incubated for two hours at 37°C while shaking at 400 rpm. After one hour the gel droplets were examined under a dissecting microscope and the *E. coli* colonies from the *S. lavendulae* condition were beginning to turn blue, indicating the presence of β-galactosidase, while those in the mutant *S. lavendulae* 11E.3 were not. After two hours the colonies in the *S. lavendulae* condition were a much stronger blue color, indicating the induction of the promoter due to the presence of a DNA-damaging agent (mitomycin C) (Figure 3A), while the mutant condition showed no sign of induction of β-galactosidase gene expression (Figure 3B).

6.3. COENCAPSULATION OF *STREPTOMYCES* AND ANTIMICROBIAL INDICATOR CELLS IN TWO GEL REGIONS

A 1 % sodium alginate solution was prepared in a mixture of 50% sterile water and F10A media. This solution was split in two and to each received 1 µl per ml of a spore stock of either *S. parvulus* or the mutant *S. lividans*.

These mixtures were allowed to degas and 10 ml were then extruded through a syringe with a 16 gauge needle into 250 ml baffled flasks containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off, and the droplets were rinsed with sterile water, and poured out into a sterile omni tray. Encapsulated *S. parvulus* and *S.lividans* were then incubated at 30°C for three days.

After three days of growth, the gel droplets were then coated with a second layer of alginate containing an *Enterococcus faecalis* (as indicator cells), thus, forming a complete screening unit. This was performed by briefly soaking the gel droplets in 135 mM calcium chloride solution. which were then transferred to a beaker of sterile water containing 20,000 cells of *E. faecalis* per ml. While stirring the mixture at 200 rpm with an overhead mixer, 1% sodium alginate was added. Once the alginate was dissolved, the mixer was slowed down to 110 rpm. The gel droplets were stirred in the alginate solution for a total of 20 minutes.

Following the mixing procedure, the alginate solution was diluted 100% with sterile water and then the gel droplets were strained out and rinsed gently with sterile water. The gel droplets were placed in a clean beaker with sterile water and stirred gently on a stir plate with a magnetic stir bar until the gel droplets were no longer touching each other. Then a concentrated calcium chloride solution was added to bring the solution with the gel droplets to 75 mM calcium chloride. The gel droplets were allowed to cure for 10 minutes. The calcium chloride solution was then strained from the gel droplets which were transferred to a 250 ml baffled flask with 50 ml F10A media. Cultures were then incubated for 24 hours at 30°C while shaking at 300 rpm.

After 24 hours the screening unit with the mutant *S. lividans* showed good *E. faecalis* growth while the screening unit containing the *S. parvulus* showed very few *E. faecalis* colonies, indicating the presence of antimicrobial activity in the screening unit. See Figure 3A.

6.4. COENCAPSULATION OF *STREPTOMYCES* AND INDICATOR CELLS IN TWO GEL REGIONS

A 1% sodium alginate solution was prepared in a mixture of 50% sterile water and F1OA media. Spores from *S. aureofaciens* were added to this mixture which was then extruded through a syringe with a 16 gauge needle into a 250 ml baffled flasks containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off and the gel droplets were rinsed with sterile water and placed on a sterile omni tray. Encapsulated *S. aureofaciens* were then incubated at 30°C for four days.

After four days, the gel droplets were coated with a second layer of alginate containing a target strain of bacteria, *Staphylococcus aureus,* thus, forming a second gel region. This was performed by briefly soaking the gel droplets in 135 mM calcium chloride solution. The gel droplets were then transferred to a beaker of sterile water containing 20,000 cells of *S. aureus* per ml and while mixing at 200 rpm with an overhead mixer, 1% sodium alginate was added. Once the alginate was dissolved, the mixer was slowed down to 110 rpm. The gel droplets were strained out and rinsed gently with sterile water. The gel droplets were placed in a clean beaker with sterile water and stirred gently on a stir plate with a magnetic stir bar until gel droplets were no longer touching each other, and then concentrated calcium chloride was added to bring the solution to 75 mM calcium chloride. The gel droplets were allowed to cure for 10 minutes. The calcium chloride solution was strained from the gel droplets which were transferred to 1 250 ml baffled flask with 50 F1OA media. Cultures were then incubated for 34 hours at 30°C while shaking at 300 rpm. After 24 hours the gel droplets were laid out on a tray and placed on a light box. Those that were showed clearing were pulled out, the alginate was dissolved in sterile 500 mM

sodium citrate and the *Streptomyces* colonies were recovered and placed on a plate of F1OA nutrient agar.

6.5. SCREENING OF ENCAPSULATED *STREPTOMYCES* COMBINATORIAL GENE EXPRESSION LIBRARY WITH INDICATOR CELLS IN A PLATE FORMAT

A 1% sodium alginate solution was prepared in R5T media. To this mixture was added a transformation solution containing a transformation of *Streptomyces lividans* protoplasts and cosmid DNA from a genomic library of *Streptomyces aureofaciens*, a producer of tetracycline. This mixture was extruded through a syringe with a 16 gauge needle into a 250 ml baffled flasks containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off and replaced with 50 ml of nutrient media (R5T). Encapsulated *S. lividans* transformed protoplasts were then incubated at 30°C with shaking at 300 rpm for 18 hours. After 18 hours, the antibiotic selection apramycin was added at a concentration of 60 μg/ml. The gel droplets were then incubated for 24 hours, after which the media was changed to F1OA. The gel droplets were then incubated for another 24 hours, at which point the media was removed and the gel droplets were rinsed briefly with sterile water and poured out into a sterile omni tray. After three days of growth, the gel droplets were overlaid with a lawn of *S. aureus* in LB top agar. The plate was then incubated at 30°C overnight and examined for zones of clearing. The ... were then tested further for biological activity and genetically analyzed for insert DNA.

6.6. COENCAPSULATION OF *STREPTOMYCES* AND RESIN CARRIERS

A 1 % sodium alginate solution was prepared in sterile water. This solution was inoculated with 1 μl per ml of a mycelial stock of *S. lavendulae* and 1% of Diaion HP20 resin. This solution was allowed to degas and 10 ml was then

extruded through a syringe with a 16 gauge needle into a 250 ml baffled flask containing 135 mM calcium chloride solution. After allowing the alginate to cure for 10 minutes, the calcium chloride was strained off and replaced with 50 ml of nutrient media (SM5A). Encapsulated *S. lavendulae* were then incubated at 30°C while shaking at 400 rpm for four days. Every 24 hours the media was removed and fresh media was added.

After four days the gel droplets were collected, isolated into 96-well plates with one gel droplet per well and the alginate was dissolved with 100 µl of 200 mM sodium citrate. The resins were removed and moved to a 96-well plate in the same order as the original. The resin can be used as a producing species in a screening unit for presentation of the chemicals to an assay system. Alternatively, the chemicals were removed from the resins with 100 µl acetone, transferred, evaporated and resuspended In 30 µl of sterile water. Chemicals can now be used for other types of assays which are less compatible with the growth conditions of *Streptomyces*.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for screening natural products comprising in the order stated:

(a) coencapsulating a producing species and an assay system in a gel droplet to form a screening unit;

(b) placing the screening unit under conditions suitable for production of natural products for an interval sufficient for the natural products produced by the producing species to contact the assay system; and

(c) detecting a signal generated by the assay system.

2. A method for screening natural products comprising the following steps in the order stated:

(a) encapsulating a producing species in a gel droplet;

(b) placing the gel droplet under culture conditions until about the time when natural products production in the producing species begins;

(c) forming around the gel droplet a second gel region comprising an assay system to form a screening unit;

(d) placing the screening unit under conditions suitable for production of natural products for an interval sufficient for the natural products to contact the assay system; and

(e) detecting a signal generated by the assay system.

3. A method for screening natural products comprising the following steps in the order stated:

(a) culturing a producing species until about the time when natural products production begins;

(b) coencapsulating the producing species and an assay system in a gel droplet to form a screening unit;

(c) placing the screening unit under conditions suitable for production of natural products for an interval sufficient for the natural products produced by the producing species to contact the assay system; and

(d) detecting a signal generated by the assay system.

4. A method for screening natural products comprising the following steps in the order stated:

(a) encapsulating a producing species in a gel droplet;

(b) placing the gel droplet under culture conditions until about the time when natural products production in the producing species begins;

(c) contacting the gel droplet with an assay system for an interval sufficient for the natural products to contact the assay system; and

(d) detecting a signal generated by the assay system.

5. A method for screening natural products comprising the following steps in the order stated:

(a) coencapsulating a producing species and a solid carrier in a gel droplet;

(b) placing the gel droplet under conditions suitable for production of natural products for an interval sufficient for the natural products produced by the producing species to sequester in the solid contact;

(c) retrieving the solid carrier;

(d) contacting the solid carrier with an assay system under conditions such that the

sequestered natural products contact the assay system; and

(e) detecting a signal generated by the assay system.

6. A method for screening compounds comprising:

(a) coencapsulating a producing species and an assay system in a gel droplet to form a screening unit, wherein the producing species is a solid carrier of a compound;

(b) placing the screening unit under conditions such that the compound contact the assay system; and

(c) detecting a signal generated by the assay system.

7. The method of claim 2 wherein the culture conditions in step (b) selects for a genetically engineered producing species.

8. The method of claim 2 wherein the producing species is a bacteria, and wherein the bacteria received foreign DNA by conjugation in the gel droplet.

9. The method of claim 2 wherein the gel droplet in step(b) is not immersed in liquid.

10. The method of claim 4 wherein the gel droplet in step(b) is not immersed in liquid.

11. A method for producing natural products comprising:

(a) encapsulating a producing species in a gel droplet,

(b) incubating the gel droplet under conditions suitable for production of natural products for an interval sufficient for the natural products

to accumulate in the gel droplet, wherein the gel droplet is not immersed in liquid; and

(c) recovering the natural products from the gel droplet.

12. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the production of natural products is induced by environmental factors or by chemicals.

13. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the gel droplet is made of alginate, carrageenan, agarose, chitosan, cellulose, pectin, or polyacrylamide.

14. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a bacteria, a fungus, a protozoan, a helminth, a parasite, an algal cell, a plant cell, an invertebrate cell, or a vertebrate cell.

15. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a *Actinomycete* or *Myxobacteria*.

16. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is Myxococcus xanthus.

17. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a *Streptomyces*.

18. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is *Streptomyces lividans, Streptomyces coelicolor, Streptomyces fradiae, Streptomyces venezuelae, Streptomyces roseosporus, Streptomyces toyocaenesis, Streptomyces griseus, Streptomyces clavuligerus, Streptomyces lavendulae, or Streptomyces arenae*.

19. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is from an environmental sample.

20. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a genetically engineered prokaryotic cell or genetically engineered eukaryotic cell.

21. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a cell comprising recombinant nucleic acid molecules encoding proteins involved in the synthesis of polyketides, aminoglycosides, nucleosides, non-ribosomal peptides, β-lactam, terpene, or shikimate-derived natural products.

22. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a genetically engineered prokarytic cell in a combinatorial gene expression library.

23. The method of claim 1, 2, 3, 4, 5, 7, 8, 9, 10 or 11 wherein the producing species is a genetically engineered eukaryotic cell in a combinatorial gene expression library.

24. The method of claim 5 or 6 wherein the solid carrier is a neutral resin bead or a polystyrene bead.

25. The method of claim 5 or 6 wherein the compound is chemically coupled to the solid carrier via a cleavable linker.

26. The method of claim 6 wherein the compound is from a combinatorial chemical library.

27. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is an indicator cell, wherein the indicator cell is selected from the group consisting of a bacterial cell, a fungal cell, a protozoa cell, a helminth cell, an algal cell, a plant cell, an invertebrate cell, a vertebrate cell, and a mammalian cell.

28. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is a pathogenic microorganism, an agricultural pest, a cell infected by an intracellular pathogen, a virus-infected cell, a tumor cell, a cell from a primary culture, or a cell from a cell line.

29. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is a whole live organism selected from the group consisting of a bacterium, a fungus, a protozoan, an agricultural pest, a helminth, a parasite, an algae, a plant, an invertebrate, and an insect.

30. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is a genetically engineered indicator cell comprising a target molecule or a reporter molecule.

31. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is a genetically engineered indicator cell comprising a promoter operably associated with a reporter gene, wherein transcription of the reporter gene is affected by the presence or activity of the natural product.

32. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the assay system is a chemical indicator that generates a signal in response to the presence or activity of the natural product.

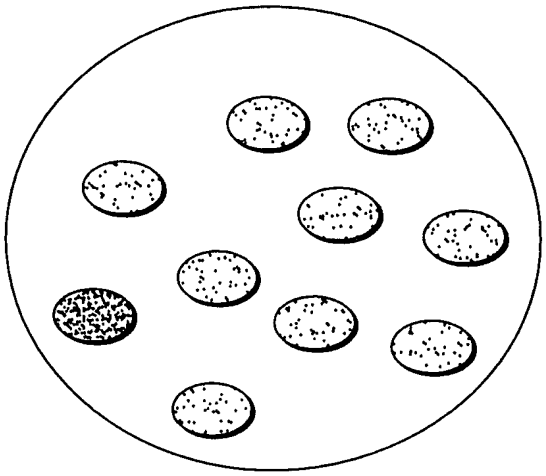

FIG. 1A

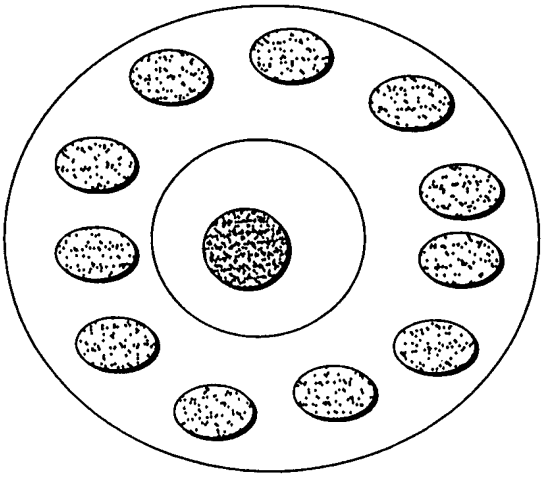

FIG. 1B

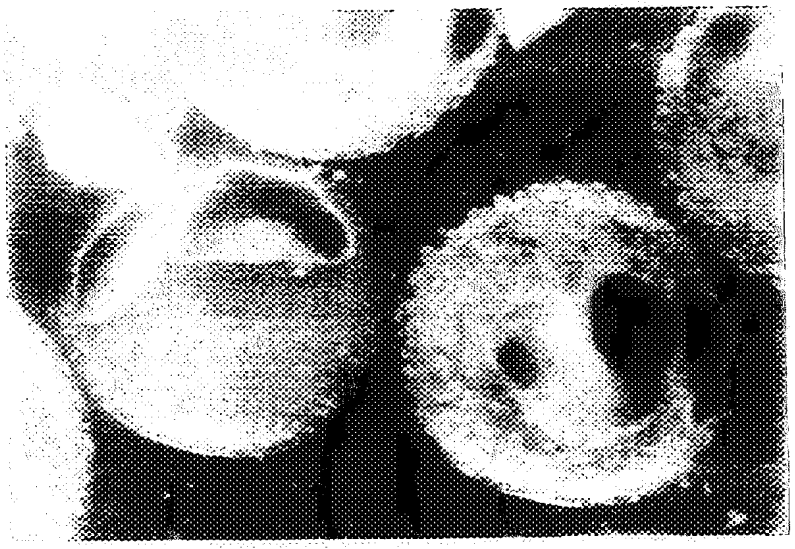

FIG. 2

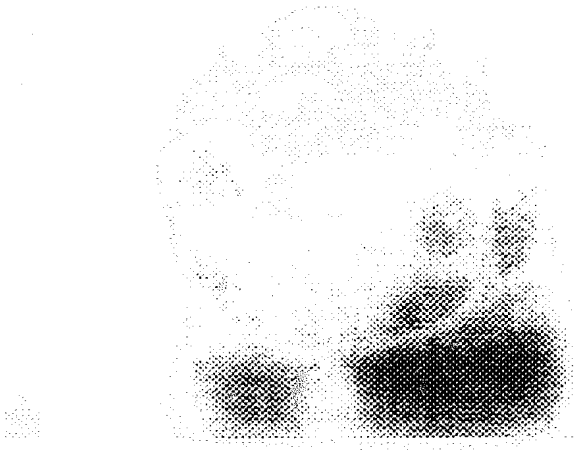

FIG.3A

FIG.3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/05462

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/549
US CL :435/7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.32, 7.33, 7.34;
436/518, 528, 531, 534, 535

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: screening assay, screening method, bacteria, microorganism, encapsulate, gel droplet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,673,566 A (GOOSEN et al) 16 June 1987, see entire document.	1-32
A	US 4,647,536 A (MOSBACH et al) 03 March 1987, see entire document.	1-32
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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